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(54) Title: PREPARATION OF STABLE FORMULATIONS OF LIPID–NUCLEIC ACID COMPLEXES FOR EFFICIENT *IN VIVO* DELIVERY

(57) Abstract

The present invention provides for lipid:nucleic acid complexes that have increased shelf life and high transfection activity *in vivo* following intravenous injection, and methods of preparing such complexes. The methods generally involve contacting a nucleic acid with an organic polycation to produce a condensed nucleic acid, and then combining the condensed nucleic acid with a lipid comprising an amphiphilic cationic lipid to produce the lipid:nucleic acid complex. This complex can be further stabilized by the addition of a hydrophilic polymer attached to hydrophobic side chains. The complex can also be made specific for specific cells, by incorporating a targeting moiety such as a Fab' fragment attached to a hydrophilic polymer.

TITLE OF THE INVENTION

PREPARATION OF STABLE FORMULATIONS OF LIPID-NUCLEIC ACID COMPLEXES FOR EFFICIENT *IN VIVO* DELIVERY

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing date of USSN 60/030,578, filed November 12, 1996.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

FIELD OF THE INVENTION

The present invention relates to the field of cationic lipid:DNA complexes ("CLDC"). In particular, the present invention relates to lipid:nucleic acid complexes that contain (1) hydrophilic polymer; (2) nucleic acid that has been condensed with organic polycations; and (3) hydrophilic polymer and nucleic acid that has been condensed with organic polycations. The lipid:nucleic acid complexes of this invention show high transfection activity *in vivo* following intravenous injection and an unexpected increase in shelf life, as determined by *in vivo* transfection activity.

BACKGROUND OF THE INVENTION

Liposomes that consist of amphiphilic cationic molecules are useful non-viral vectors for gene delivery *in vitro* and *in vivo* (reviewed in Crystal, *Science* 270: 404-410 (1995); Blaese *et al., Cancer Gene Ther.* 2: 291-297 (1995); Behr *et al., Bioconjugate Chem.* 5: 382-389 (1994); Remy *et al., Bioconjugate Chem.* 5: 647-654 (1994); and Gao *et al., Gene Therapy* 2: 710-722 (1995)). In theory, the positively charged liposomes complex to negatively charged nucleic acids via electrostatic interactions to form lipid:nucleic acid complexes. The lipid:nucleic acid complexes have several advantages as gene transfer vectors. Unlike viral vectors, the lipid:nucleic acid complexes can be

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development of lipid-mediated gene therapy.

SUMMARY OF THE INVENTION

The present invention provides a novel method of preparing cationic lipid:nucleic acid complexes that have increased shelf life. In one embodiment, these complexes are prepared by contacting a nucleic acid with an organic polycation, to produce a condensed or partially condensed nucleic acid. The condensed nucleic acid is then combined with an amphiphilic cationic lipid plus a neutral helper lipid such as cholesterol in a molar ratio from about 2:1 to about 1:2, producing the lipid:nucleic acid complex. Optionally, a hydrophilic polymer is subsequently added to the lipid:nucleic acid complex. Alternatively, the hydrophilic polymer is added to a lipid:nucleic acid complex comprising nucleic acid that has not been not condensed. These lipid:nucleic acid complexes have an increased shelf life, *e.g.*, when stored at 22°C or below, as compared to an identical lipid:nucleic acid complex in which the nucleic acid component has not been contacted with the organic polycation and/or in which the lipid:nucleic acid complex has not been contacted with a hydrophilic polymer.

In a particularly preferred embodiment, the polycation is a polyamine, more preferably a polyamine such as spermidine or spermine.

In another preferred embodiment, the lipid:nucleic acid complexes are prepared by combining a nucleic acid with an amphiphilic cationic lipid and then combining the complex thus formed with a hydrophilic polymer. This lipid:nucleic acid complex has an increased shelf life, *e.g.*, when stored at 22°C or below as compared to an identical complex that has not been combined with the hydrophilic polymer.

In one embodiment, the hydrophilic polymer is selected from the group consisting of polyethylene glycol (PEG), polyethylene glycol derivatized with phosphatidyl ethanolamine (PEG-PE), polyethylene glycol derivatized with tween, polyethylene glycol derivatized with distearoylphosphatidyl-ethanolamine (PEG-DSPE), ganglioside G_{M1} and synthetic polymers.

In one embodiment, the lipid:nucleic acid complex is lyophilized.

In any of the methods and compositions of this invention, the nucleic acid can be virtually any nucleic acid, *e.g.*, a deoxyribonucleic acid

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has increased shelf life when stored at about 4°C.

In one particularly preferred embodiment, the method of increasing the shelf life of the lipid:nucleic acid complex includes the steps of combining an expression cassette with spermidine or spermine with an amphiphilic cationic lipid, and a Fab' fragment of an antibody attached to a polyethylene glycol derivative. In another particularly preferred embodiment, includes the steps of combining an expression cassette with an amphiphilic cationic lipid, and a Fab' fragment of an antibody attached to a polyethylene glycol derivative so that the complex has increased shelf life when stored at about 4°C.

This invention also provides for a method of transfecting a nucleic acid into a mammalian cell, the method comprising contacting the cell with any one of the lipid:nucleic acid complexes prepared as described above. In one embodiment, the method uses systemic administration of a lipid:nucleic acid complex into a mammal. In a preferred embodiment, the method of transfecting uses intravenous administration of the lipid:nucleic acid complex into a mammal. In a particularly preferred embodiment, the method comprises contacting a specific cell that expresses a ligand that recognizes the Fab' fragment.

In yet another embodiment, this invention also provides for pharmaceutical composition comprising the lipid:condensed nucleic acid complex described above. The pharmaceutical compositions comprise a therapeutically effective dose of the lipid:nucleic acid complex and a pharmaceutically acceptable carrier or excipient.

In yet another embodiment, the invention also provides a kit for preparing a lipid:nucleic acid complex, the kit comprising a container with a liposome; a container with a nucleic acid; and a container with a hydrophilic polymer, wherein the liposome and the nucleic acid are mixed to form the lipid:nucleic acid complex and wherein the lipid:nucleic acid complex is contacted with the hydrophilic polymer. In a preferred embodiment, the hydrophilic polymer is derivatized with a targeting moiety, preferably an Fab' fragment. In another preferred embodiment, the nucleic acid is condensed.

This invention also provides for a lipid:condensed nucleic acid complex prepared using the method of increasing shelf life using nucleic acid

Figure 5A and 5B illustrate *in vitro* transfection of cell lines with immunolipid:DNA complexes. The samples are as follows: (1) DDAB/DOPE (1:1), producing cationic liposomes complexed with DNA only; (2) DDAB/DOPE (1:1) with 1% PEG-PE derivatized with maleimide at the ultimate position of PEG, producing liposomes with the steric stabilization component added after complexation with the DNA; and (3) DDAB/DOPE (1:1) with 1% PEG-PE derivatized with the Fab' fragment of a humanized anti-Her-2 antibody attached to the ultimate position of PEG via the free thiol group to the maleimide residue.

DEFINITIONS

The following abbreviations are used herein: Chol, cholesterol; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; SM, sphinogmyelin; M-DPE, maleimide derivatized dipalmityolethanolamine; PBS, phosphate buffered saline; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PEG-PE, polyethylene glycol derivatized phosphatidylethanolamine, DC-chol, 3β [N-(N', N'-dimethylaminoethane) carbanoyl]-cholesterol; DDAB, Dimethyidioctadecylammoniumbromide; DMEPC, Dimyristoylglycero-3-ethyl phosphocholine; DODAP, Dioleoyl-3-dimethylammonium propane; DOEPC, Dioleoylglycero-3-ethyl phosphocholine; DOGS, N,N-Dioctadecylamidoglycyl spermine; DOPE, Dioleoylphosphatidylethanolamine; DOTAP, Dioleoyl-3-trimethylammonium propane; DOTMA, N-[2,3-(dioleyloxy) propyl] N,N,N-trimethyl ammonium bromide; DSPE, Distearoylphosphatidylethanolamine; PEG-PE, N-[ω-methoxypoly(oxyethylene)-αoxycarbonyl]-DSPE; POEPC, Palmitoyloleoylglycero-3-ethyl phosphocholine.

The term "amphiphilic cationic lipid" is intended to include any amphiphilic lipid, including synthetic lipids and lipid analogs, having hydrophobic and polar head group moieties, a net positive charge, and which by itself can form spontaneously into bilayer vesicles or micelles in water, as exemplified by phospholipids. The term also includes any amphiphilic lipid that is stably incorporated into lipid bilayers in combination with phospholipids with its hydrophobic moiety in contact with the interior,

contain other compounds such as a polycation that are in contact with the nucleic acid of the complex, producing condensed nucleic acid, and hydrophilic polymers such as PEG and derivatized PEG.

The terms "immunoliposome" and "immunolipid:nucleic acid complex" refer to a liposome or lipid:nucleic acid complex bearing an antibody or antibody fragment that acts as a targeting moiety enabling the lipid:nucleic acid complex to specifically bind to a particular "target" molecule that may exist in solution or may be bound to the surface of a cell. Where the target molecule is one that is typically found in relative excess (*e.g.*, ≥ 10-fold) and in association with a particular cell type or alternatively in a multiplicity of cell types all expressing a particular physiological condition the target molecule is said to be a "characteristic marker" of that cell type or that physiological condition. Thus, for example, a cancer may be characterized by the overexpression of a particular marker such as the HER2 (c-*erb*B-2/*neu*) proto-oncogene in the case of breast cancer.

A "hydrophilic polymer" as used herein refers to long chain highly hydrated flexible neutral polymers attached to lipid molecules. Examples include, but are not limited to polyethylene glycol (PEG), polyethylene glycol derivatized with phosphatidyl ethanolamine (PEG-PE), polyethylene glycol derivatized with tween, polyethylene glycol derivatized with distearoylphosphatidylethanolamine (PEG-DSPE), ganglioside G_{M1} and synthetic polymers. Such polymers typically have a molecular weight in the range of 1000-10,000. Preferably, the molecular weight for PEG is approximately 2000.

"Transfection" refers to contacting a living cell with a nucleic acid, for example, as part of a lipid:nucleic acid complex.

"Transfection activity" refers to the efficiency of introducing a nucleic acid into a living cell. Transfection efficiency may be measured by determining the amount of expression of a reporter gene that has been transfected into the cell as part of a lipid:nucleic acid complex, for example, by fluorescent or functional assays.

The terms "condensed nucleic acid" and "partially condensed nucleic acid" are used to refer to a nucleic acid that has been contacted with an organic cation for example, polyamines, including spermine and

related structural variants or synthetic analogs thereof). Thus, the term refers to a nucleotide polymer in which the nucleotides and the linkages between them are naturally occurring (DNA or RNA), as well as various analogs, for example and without limitation, peptide-nucleic acids (PNAs), phosphoramidates, phosphorothioates, methyl phosphonates, 2-O-methyl ribonucleic acids, and the like.

The term "mole percent" when referring to the percentage of hydrophilic polymer in a liposome is expressed relative to the cationic lipid in the liposome unless otherwise stated. Thus, for example, in a liposome comprising a ratio of DDAB to cholesterol (Chol) of 100:100, a 4 mole percent of hydrophilic polymer (*e.g.,* PEG) would represent a ratio of DDAB:Chol:PEG of about 100:100:4.

The term "identical" refers to a composition that is formed using the same compounds as another composition, where the compositions do not differ in a statistically significant manner.

The term "systemic administration" refers to a method of administering a compound or composition to a mammal so that the compound or composition is delivered to many sites in the body via the circulatory system.

DETAILED DESCRIPTION

This invention provides methods of increasing the shelf life of cationic lipid:nucleic acid complexes, and *in vivo* and/or *in vitro* transfection efficiency of these complexes. Such complexes have attracted considerable interest as a means of delivering nucleic acids expressing various therapeutic polypeptides as a means of delivering therapeutic (*e.g.,* antisense) nucleic acids themselves. Unfortunately, it has been difficult to maintain and store homogeneous lipid:nucleic acid complexes suitable for *in vivo* administration. The complexes tend to aggregate rapidly or decompose within a relatively short time. This instability has required use of these complexes within a short period of time after preparation, often as little as 30 minutes up to a few hours. Thus, for example, in recent clinical trials using cationic lipids as a carrier for DNA delivery, the DNA and lipid components were mixed at the bedside and used immediately (Gao *et al.*, *Gene Therapy* 2: 710-722 (1995)).

polymer. This invention also include the lipid:nucleic acid complexes thus prepared.

I. Cationic lipid:nucleic acid complexes

As explained above, this invention provides methods of increasing the storage life (shelf life) of lipid:nucleic acid complexes. In a preferred embodiment the complexes are formed by combination of a nucleic acid with a liposome. It is recognized, however, that the lipids need not be provided as a liposome. It is also recognized that after complexation, the lipid:nucleic acid complex may no longer exist as a true vesicle and therefore is not generally regarded as a liposome. The preparation of lipid:nucleic acid complexes is well known to one of skill in the art (*see, e.g.*, reviewed in Crystal, *Science* 270: 404-410 (1995); Blaese *et al., Cancer Gene Ther.* 2: 291-297 (1995); Behr *et al., Bioconjugate Chem.* 5: 382-389 (1994); Remy *et al., Bioconjugate Chem.* 5: 647-654 (1994); and Gao *et al., Gene Therapy* 2: 710-722 (1995)). The various components and construction of the stabilized lipid:nucleic acid complexes of the invention are described in detail below.

A. Amphiphilic cationic lipids

As indicated above, the methods of this invention involve complexing a cationic lipid with a nucleic acid. The term "cationic lipid" refers to any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, DODAC, DOTMA, DDAB, DOTAP, DC-Chol and DMRIE. Additionally, a number of commercial preparations of cationic lipids are available which can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic lipids comprising DOGS in ethanol from Promega Corp., Madison, Wisconsin, USA).

The cationic lipid can be used alone, or in combination with a "helper" lipid. Preferred helper lipids are non-ionic or uncharged at

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used for expression of recombinant proteins in prokaryotic or eukaryotic cells can be used.

The expression vectors typically have a transcription unit or expression cassette that contains all the elements required for the expression of the nucleic acid in the host cells. A typical expression cassette contains a promoter operably linked to the DNA sequence encoding a protein. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

In the expression cassette, the nucleic acid sequence of interest can be linked to a sequence encoding a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. The expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region can be obtained from the same gene as the promoter sequence or can be obtained from a different gene.

For more efficient translation in mammalian cells of the mRNA encoded by the structural gene, polyadenylation sequences are also commonly added to the expression cassette. Termination and polyadenylation signals that are suitable for the present invention include those derived from SV40, or a partial genomic copy of a gene already resident on the expression vector.

In addition to the expression cassette, many expression vectors optimally include enhancer elements that can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus, human or murine cytomegalovirus, the long terminal repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus, and HIV (*see Enhancers and Eukaryotic Expression* (1983)).

In addition to the recombinant nucleic acids discussed above, synthetic nucleic acids or oligonucleotides can also be used in the invention.

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liposomes. However, condensing nucleic acid prior to lipid complex formation produced the surprising result of increased shelf life for lipid:nucleic acid complexes, as measured by transfection efficiency. The lipid:nucleic acid complexes formed with such pretreatment were stable at a lower ratio of lipid to DNA without aggregation. Organic polycations such as polyamines, polyammonium molecules, and basic polyamino acids, and their derivatives are used to condense the nucleic acid prior to lipid complex formation. A preferred embodiment uses polyamines such as spermidine and spermine to condense the nucleic acid (*see, e.g.*, Example 1).

D. Hydrophilic polymer

It has been established recently that PEG-PE incorporation in liposomes produces steric stabilization resulting in longer circulation times in blood (Allen *et al.*, *Biochim. Biophys. Acta* 1066: 29-36 (1991); Papahadjopoulos *et al.*, *Proc. Natl. Acad. Sci. USA* 88: 11460-11464 (1991)). In the present invention, inserting PEG-PE (*e.g.*, 1% of total lipid) into the freshly formed lipid:nucleic acid complexes prevents the complexes from aggregating during storage. It was a surprising discovery, however, that the incorporation of PEG-PE did not inhibit transfection activity *in vivo* and also that the *in vitro* transfection activity, which was inhibited, was regained by the incorporation of Fab' fragment conjugated at the end of the PEG-PE. The presence of hydrophilic polymers in the lipid:nucleic acid complex provides increased shelf life, as measured by transfection efficiency after storage. Thus, it is desirable to add a hydrophilic polymer such as polyethylene glycol (PEG)-modified lipids or ganglioside G_{M1} to the liposomes. PEG may also be derivatized with other amphipathic molecules such as fatty acids, sphingolipids, glycolipids, and cholesterol. Addition of such components prevents liposome aggregation during coupling of the targeting moiety to the liposome. These components also provides a means for increasing circulation lifetime of the lipid:nucleic acid complexes.

A number of different methods may be used for the preparation of PEG for incorporation into liposomes. In one preferred embodiment, PEG is incorporated as PEG derivatized phosphatidylethanolamine (PEG-PE) or PEG derivatized distearoyl phosphatidylethanolamine (PEG-DSPE). Methods

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immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

The basic immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies may exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. In particular, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'$_2$, a dimer of Fab' which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'$_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the F(ab)'$_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see Fundamental Immunology*, W.E. Paul, ed., Raven Press, N.Y. (1993) for more antibody fragment terminology). While the Fab' fragment is defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology.

The Fab' fragments used in the present invention may be derived from antibodies of animal (especially mouse or rat) or human origin or may be chimeric (Morrison *et al.*, *Proc Natl. Acad. Sci. USA* 81: 6851-6855 (1984)) or humanized (Jones *et al.*, *Nature* 321: 522-525 (1986), and published UK patent application No. 8707252).

The Fab' fragment is selected to specifically bind to a molecule or marker characteristic of the surface of the cells to which it is desired to deliver the contents of the cationic lipid:nucleic acid complex. A molecule is

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(IL7), granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), erythropoietin, interleukin 13 receptor (IL13R), and the like. One of skill in the art recognizes that the term growth factor as used herein generally includes cytokines and colony stimulating factors.

Particularly preferred markers are found in the HER family of growth factor receptors. More specifically HER1, HER2, HER3 and HER4 are more preferred with HER2 most preferred. The HER receptors comprise protein tyrosine kinases that themselves provide highly specific antibody targets. Thus, in one embodiment, the P185 tyrosine kinase of HER2 provides a most preferred target for the Fab' fragment of the utilized in the immunolipid:nucleic acid complexes of the present invention.

It will be appreciated that the characteristic marker need not be a naturally occurring marker, but rather may be introduced to the particular target cell. This may be accomplished by directly tagging a cell or tissue with a particular marker (*e.g.*, by directly injecting the particular target tissue with a marker, or alternatively, by administering to the entire organism a marker that is selectively incorporated by the target tissue. In one embodiment, the marker may be a gene product that is encoded by a nucleic acid in an expression cassette. The marker gene may be under the control of a promoter that is active only in the particular target cells. Thus introduction of a vector containing the expression cassette will result in expression of the marker in only the particular target cells. One of skill in the art will recognize that there are numerous approaches utilizing recombinant DNA methodology to introduce characteristic markers into target cells.

In one preferred embodiment, the targeting moiety will specifically bind products or components of a growth factor receptor, in particular products of the HER2 (c-*erb*B-2, *neu*) proto-oncogene. It is particularly preferred that the targeting moiety bind the growth factor receptor-tyrosine kinase encoded by HER2, protein $p185^{HER2}$, which is commonly overexpressed in breast cancers (Slamon *et al., Science* 235: 177-182 (1987). Other suitable targets for the targeting moiety include, but are not limited to EGFR (HER1), HER3, and HER4, combinations of these receptors, and other markers associated with cancers. Other antibodies of

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& Bangham, *Biochim. Biophys. Acta* 443: 629-634 (1976); Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* 76: 3348-3352 (1979); Hope *et al.*, *Biochim. Biophys. Acta* 812: 55-65 (1985); Mayer *et al.*, *Biochim. Biophys. Acta* 858: 161-168 (1986); Williams *et al.*, *Proc. Natl. Acad. Sci. USA* 85: 242-246 (1988), *Liposomes*, ch. 1 (Ostro, ed., 1983); and Hope *et al.*, *Chem. Phys. Lip.* 40: 89 (1986). Suitable methods include, *e.g.*, sonication, extrusion, high pressure/homogenization, microfluidization, detergent dialysis, calcium-induced fusion of small liposome vesicles, and ether-infusion methods, all well known in the art. One method produces multilamellar vesicles of heterogeneous sizes. In this method, the vesicle-forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form. This film is covered with an aqueous buffered solution and allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate.

In a preferred embodiment, mostly unilammellar liposomes are produced by the reverse phase evaporation method of Szoka & Papahadjopoulos, *Proc. Natl. Acad. Sci. USA*, 75: 4194-4198 (1978).

Unilamellar vesicles are generally prepared by sonication or extrusion. Sonication is generally performed with a bath-type sonifier, such as a Branson tip sonifier at a controlled temperature as determined by the melting point of the lipid. Extrusion may be carried out by biomembrane extruders, such as the Lipex Biomembrane Extruder. Defined pore size in the extrusion filters may generate unilamellar liposomal vesicles of specific sizes. The liposomes may also be formed by extrusion through an asymmetric ceramic filter, such as a Ceraflow Microfilter, commercially available from the Norton Company, Worcester MA.

Following liposome preparation, the liposomes that have not been sized during formation may be sized by extrusion to achieve a desired size range and relatively narrow distribution of liposome sizes. A size range

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III. Formation of lipid:nucleic acid complexes

It was a discovery of this invention that stabilized lipid:nucleic acid complexes (e.g., having condensed nucleic acid and/hydrophilic polymer) tended not to form visible large aggregates and had increased transfection efficiency and shelf life. Nucleic acid/liposome ratios for preparing lipid:nucleic acid complexes that do not form visible large aggregates can be determined by one of skill in the art. Typically, the ratio is determined by mixing fixed amounts of a nucleic acid, *e.g.*, a plasmid, to various amounts of liposomes (*see* Example 1). In general, lipid:nucleic acid complexes are formed by pipetting the nucleic acid (*e.g.*, plasmid DNA) into a liposome suspension of equal volume and mixing rapidly. Routinely, liposomes containing 5-15 nmole of a lipid such as DDAB or DOPE (as described above) form a complex with 1 μg plasmid, without forming visible large aggregates. Inspection for visible large aggregates is typically performed without the aid of a microscope. The endpoint of the titration of the amounts of lipid and nucleic acid is also achieved by assaying for increased transfection efficiency, either *in vitro* or *in vivo*, as compared to a non-stabilized control (as described below).

To keep the lipid:nucleic acid complexes from forming large aggregates and losing transfecting activity with time, two approaches are taken: (1) incorporating a small amount of a hydrophilic polymer such as PEG-PE (approx. 1 % mole ratio) into lipid:nucleic acid complexes within a few minutes after their preparation; and/or (2) condensing the nucleic acid with a polycation such as a polyamine (*e.g.*, approximately 0.05 to 5.0 nmole of spermidine per μg DNA) prior to mixing with the liposomes. The optimal amount of the polyamines and hydrophilic polymer can be determined by one of skill in the art by titrating the polyamine or hydrophilic polymer with the nucleic acid so that the formed complexes do not form large, *e.g.*, visible, aggregates. The size of these lipid:nucleic acid complexes can be estimated by dynamic light scattering to be in the range of 410 ± 150 nm. The endpoint of the titration is also achieved by assaying for increased transfection efficiency either *in vitro* or *in vivo*, as compared to a non-stabilized control (as described below).

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luciferase, β-galactosidase, chloramphenicol acetyl transferase (CAT), human growth hormone (hGH), and the green fluorescent protein (GFP) are preferred examples of genes that can be used in assays to determine transfection efficiency. In one embodiment of the invention, luciferase can be used as a reporter gene to determine transfection efficiency.

Transfection efficiency of a reporter gene can be determined with an assay that is appropriate for the reporter gene in use. Such assays are known to those skilled in the art. For example, the HGH reporter assay is immunologically based and employs commercially available radioimmunoassay kits. In a preferred embodiment of the invention, the luciferase assay is used to detect transfection and expression of the luciferase reporter gene. The luciferase assay is preferred because it is highly sensitive and does not use radioactivity. A luminometer can be used to measure the luciferase enzyme activity, as described in Example 1.

Gene therapy provides methods for combating chronic infectious diseases such as HIV infection, as well as non-infectious diseases such as cancer and birth defects (*see generally* Anderson, *Science* 256: 808-813 (1992); Yu *et al.*, *Gene Ther.* 1: 13-26 (1994)). Gene therapy can be used to transduce cells with either an *ex vivo* or an *in vivo* procedure. *Ex vivo* methods for gene therapy involve transducing the cell outside of the mammal with a lipid:nucleic acid complex of this invention, and introducing the cell back into the organism. The cells can be hematopoietic stem cells isolated from bone marrow or other cells that can be transfected by lipid:nucleic acid complexes.

In humans, hematopoietic stem cells can be obtained from a variety of sources including cord blood, bone marrow, and mobilized peripheral blood. Purification of $CD34^+$ cells can be accomplished by antibody affinity procedures (*see* Ho *et al.*, *Stem Cells* 13 (suppl. 3): 100-105 (1995); *see also* Brenner, *J. Hematotherapy* 2: 7-17 (1993)). Cells can also be isolated and cultured from patients. Alternatively, the cells used for *ex vivo* procedures can be those stored in a cell bank (*e.g.*, a blood bank). The advantage to using stem cells is that they can be differentiated into other cell types *in vitro*, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for

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being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, *etc*. Additionally, the lipid:nucleic acid complex suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

The concentration of lipid:nucleic acid complexes in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, immunolipid:nucleic acid complexes composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration. The amount of lipid:nucleic acid complex administered will depend upon the particular Fab' used, the disease state being treated, and the judgement of the clinician. Generally the amount of lipid:nucleic acid complexes administered will be sufficient to deliver a therapeutically effective dose of the nucleic acid. The quantity of lipid:nucleic acid complex necessary to deliver a therapeutically effective dose can be determined by one skilled in the art. Typical lipid:nucleic acid complex dosages will generally be between about 0.01 and about 50 mg nucleic acid per kilogram of body weight, preferably between about 0.1 and about 10 mg nucleic acid/kg body weight, and most preferably between about 2.0 and about 5.0 mg nucleic acid/kg of body weight. For administration to mice, the dose is typically 50-100 μg per 20 g mouse.

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Alternatively, sequences or subsequences may be amplified by a variety of DNA amplification techniques including, but not limited to polymerase chain reaction (PCR) (Innis *et al., PCR Protocols: A guide to Methods and Application* (1990)), ligase chain reaction (LCR) (*see* Wu & Wallace, *Genomics* 4: 560 (1989); Landegren *et al., Science* 241: 1077 (1988); Barringer *et al., Gene* 89: 117 (1990), transcription amplification (Kwoh *et al., Proc. Natl. Acad. Sci. USA* 86: 1173 (1989)), and self-sustained sequence replication (Guatelli *et al., Proc. Nat. Acad. Sci. USA* 87: 1874 (1990)).

In a particularly preferred embodiment, transfection is evaluated by detecting the presence or absence or quantifying a gene product in one or more tissues. Any gene that expresses an easily assayable product will provide a suitable indicator for the present assay. Suitable reporter genes are well known to those of skill in the art. They include, but are not limited to, bacterial chloramphenicol acetyl transferase (CAT), beta-galactosidase, or luciferase (*see, e.g.*, Alam *et al., Analytical Biochemistry* 188: 245-254 (1990)). One particularly preferred reporter gene is the Fflux gene as illustrated in the Examples.

VIII. Assaying shelf life

As indicated above, the term "shelf life" is used herein to refer to the period of time the lipid:nucleic acid complex can be stored (under defined conditions *e.g.*, in a buffer at 4°C) before losing its biological activity. The biological activity assayed for determination of shelf life in the present invention is the ability of the lipid:nucleic acid complex to transfect mammalian cells *in vivo* after intravenous administration.

In a preferred embodiment the shelf life is determined by storing the lipid:nucleic acid complexes for varying periods of time, injecting one or more test animals with the complex and assaying selected tissues in the animal for transfection (*e.g.*, expression of a reporter gene) as described above and as illustrated in the examples.

It will be appreciated that shelf life can be expressed in absolute terms, *i.e.*, the length of time the composition can be stored before loosing activity. Alternatively, shelf life can be expressed in relative terms by reference to a different composition. Thus, for example, when the subject

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nucleic acid (condensed or uncondensed), hydrophilic polymers, hydrophilic polymers derivatized with targeting moieties such as Fab' fragments, and instructions. A wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit and the particular needs of the user. For example, the kit may contain any one of a number of targeting moieties for targeting the complex to a specific cell type, as described above.

The kit may optionally include instructional materials containing directions (*i.e.*, protocols) providing for the use of the cationic lipid:nucleic acid complex for transfecting cells *in vivo*, *ex vivo*, or *in vitro*. Typically, the instruction materials describe the procedure for preparing the lipid:nucleic acid complex from liposomes and nucleic acid, as described above. The instruction materials also describe how to mix the hydrophilic polymer with the lipid:nucleic acid complex. Additionally, the instruction materials can describe procedures for transfecting cells with the lipid:nucleic acid complex.

While the instructional materials typically comprise written or printed materials, they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*, CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXAMPLES

The invention is illustrated by the following examples. These examples are offered to illustrate, but not to limit the present invention.

Example 1: Preparation of stable lipid:plasmid DNA complexes for *in vivo* gene delivery

A. Materials and methods

1. Lipids & other reagents

DOPE was purchased from Avanti (Alabaster, AL). Highly purified Cholesterol was obtained from Calbiochem (San Diego, CA). DDAB and dextran (M.W. 40,000) were purchased from Sigma (St. Louis, MO).

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4. Preparation of transfection complexes

Prior to the transfection experiments, the optimal DNA/liposome ratio for forming complexes which were not large aggregates was determined by mixing fixed amount plasmid to various amount of liposomes. In general, the transfection complexes were formed by pipetting plasmid into liposome suspension of equal volume and mixing rapidly. Routinely, liposomes containing 8-12 nmole of DDAB could complex with 1 μg plasmid without forming visible large aggregates. Such complexes have excess positive charge, but still tend to aggregate with time during storage at 4°C and lose transfection activity in 4 days. For *in vitro* experiments, which called for much dilute complexes, cationic lipid:plasmid DNA complexes ("CLDC") at 5 nmole DDAB per μg DNA were used. To keep the lipid:plasmid DNA complexes from forming large aggregates and losing transfecting activity with time, two approaches were taken: (1) incorporating a small amount of PEG-PE (approx. 1 % mole ratio) into lipid:plasmid DNA complexes within a few minutes after their preparation; and/or (2) condensing plasmid with polyamines (*e.g.*, 0.05 to 5.0 nmole of spermidine per μg DNA) prior to mixing with liposomes. The optimal amount of the polyamines was determined by titrating polyamines to DNA before forming large aggregates. The size of these complexes was estimated by dynamic light scattering to be in the range of 410 ± 150 nm.

5. Assay of reporter gene expression

Purified luciferase was purchased from Boehringer Mannheim as a standard for calibrating the luminometer and constructing a control standard for the relative specific activity of luciferase. Reporter gene expression in a tissue extract was presented in nanogram quantities by converting relative light unit measured from a luminometer into weight unit according to a standard curve. Luciferase expressed in cells or tissues was extracted with chemical cell lysis. Effective lysis buffer consisted of 0.1 M potassium phosphate buffer at pH 7.8, 1 % Triton X-100, 1 mM DTT and 2 mM EDTA.

Female CD1 mice (4-6 weeks old, weighing approx. 25 g) were obtained from Charles River Laboratory. Mice received lipid:plasmid DNA

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basis of *in vitro* studies, that DOPE may facilitate the cytoplasmic delivery via membrane fusion once positively charged lipid:plasmid DNA complexes are bound to the cell membrane (Zhou *et al.*, *Biochim. Biophys. Acta* 1189: 195-203 (1994)). Even though Friend *et al.* did not obtain any morphological evidence that the DOTMA/DOPE lipid:plasmid DNA complexes fuse directly with the plasma membrane, they do not exclude the possibility of fusion events (Friend *et al.*, *Biochim. Biophys. Acta* 1278: 41-50 (1996)). They suggested that the complexes are endocytosed and the cationic lipids disrupt the endosomal/lysosomal membranes and then facilitate an escape of the DNA complexes into the cytoplasm and eventually into the nucleus.

Contrary to most expectations, the "helper" role of DOPE established from *in vitro* studies is not evident for *in vivo* gene delivery following i.v. injection of the complexes. When DOPE was included in DDAB cationic liposomes, the *in vivo* gene transfection was inhibited. This DOPE-dependent inhibition is shown in Figure 1. Cholesterol, not DOPE, was found to be effective as "helper" lipid for *in vivo* gene delivery. There was a ten-fold reduction in luciferase expression in mouse lungs when half of the cholesterol was replaced with DOPE. The *in vivo* results of DDAB and other cationic liposomes are not consistent with the general assumption that DOPE is a suitable "helper" lipid. On the contrary, DOPE in cationic lipid:plasmid DNA complexes attenuates the *in vivo* transfection to such a great degree that DOPE is considered as an inhibitory agent in formulations for *in vivo* gene delivery. Cholesterol has been chosen for *in vivo* studies in recent published reports (Liu *et al.*, *J. Biol. Chem.* 270: 24864-70 (1995); Solodin *et al.*, *Biochemistry* 34: 13537-44 (1995)) in which the authors do not elaborate on how and why they selected different "helper" lipids for their experimental designs, *i.e.* DOPE for *in vitro* and cholesterol for *in vivo* studies. Stabilization of anionic and neutral liposomes in blood by cholesterol has been known for a long time (Mayhew *et al.*, *Cancer Treat. Rep.* 63: 1923-1928)1979)). It is therefore obvious that for systemic gene delivery, one has to consider the stability of lipid:plasmid DNA complexes in blood, various components of which are known to react with macromolecular complexes. In fact, the preliminary study of various formulations of lipid:plasmid DNA complexes using freeze-fracture electron microscopy has shown that the

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different cationic lipids which was screened recently. Preliminary results of expression in mouse lung following i.v. injection indicated that DOTMA/Chol, DOTAP/Chol, MMCE/Chol and ESPM/Chol gave 10-100% transfection activity of DDAB/Chol, DOGS/Chol, POEPC/Chol, LYSPE/DOPE and DC-Chol/DOPE gave 1-10% of DDAB/Chol. DOEPC/Chol, DMEPC/Chol, DODAP/Chol and DDAB/DOPE did not give any measurable activity.

In parallel with the transfection studies, the morphology of these complexes in serum and in cell medium was examined by freeze-fracture electron microscopy. When examined in 50% mouse serum (10 minute incubation time), non-stabilized, one day old CLDC are as small as they are in buffer at low ionic strength (100-250 nm) but show very few protrusions. Six day old, non stabilized CLDC incubated in 50% mouse serum appeared as densely packed aggregates of spherical particles, with a high number of attached particles. Such formulations have lost all of their *in vivo* transfection activity within 4 days. Residual fibrillar protrusions are not observed.

PEG-PE stabilized CLDC incubated in 50% mouse serum were small (100-200 nm) even at six days. Similarly, CLDC prepared with condensed DNA were also quite small even after six days of storage. Specifically, the CLDC were shaped like "map pins" that were structurally stable in the presence of serum.

After incubation in cell medium (RPMI-1640 with 10% FCS), non-stabilized six day old CLDC were morphologically similar to those incubated in mouse serum, as described above. These complexes, however, were more loosely packed and showed no fibrillar protrusions. Similar morphology was observed with PEG-PE stabilized CLDC and condensed DNA CLDC incubated in cell medium.

2. Increasing shelf life for transfection activity

The relationship between structural stability and transfection activity of lipid:plasmid DNA complexes has not been detailed in the published reports so far. Screening procedures have been established to avoid large aggregates of lipid:plasmid DNA complexes by changing the ratio of DNA to lipid from net negatively charged to positively charged. Lipid:plasmid

polyamine-treated lipid:plasmid DNA complexes, when compared to that of the samples which were not pretreated with polyamines and used immediately after complexes were formed. A different approach to obtain stable cationic lipid/DNA complexes by complexing plasmid with lipid in lipid-detergent micelles was published recently (Hofland *et al.*, *Proc. Natl. Acad. Sci. USA* 93: 7305-7309 (1996)). However, only 30% of the transfection efficiency was maintained by such complexes in 15% serum for *in vitro,* and no *in vivo* results were reported.

iii. Increasing transfection stability: lyophilization

Finally, conditions have been established for the stabilization of lipid:plasmid DNA complexes by lyophilization. Liposomes composed of DDAB/Chol suspended by sonication in 5% (w/v) of dextran in water, when mixed with DNA in 1:10 ratio (μg DNA per nmole DDAB) as described in methods, could be lyophilized without loss of activity. The final concentration of dextran in which lipid:plasmid DNA complexes were formed was 8% (w/v). The lyophilized preparations were reconstituted by adding distilled water and their transfection activity in the lungs of mice after i.v. injection was measured by luciferase reporter gene expression. Freezing and thawing the reconstituted preparation did not affect the activity (usually 1-2 ng luciferase protein per mg tissue protein).

Several of the cationic lipid:plasmid DNA complexes described herein are stable and can give consistent *in vivo* transfection activity (ranging from 0.5 to 2 ng luciferase per mg tissue protein) even after long storage at 4°C or lyophilization. Formulations containing cholesterol as the "helper" lipid generate much higher *in vivo* transfection efficiency. Stabilizing the complex structure by PEG-PE maintains the complex activity in storage and may prolong the circulation time in blood for targeting to specific tissues. Condensing the DNA with polyamines before lipid complexation enhances *in vitro* storage and levels of activity *in vivo*. The methodical approach for producing stable formulations of lipid:plasmid DNA complexes exhibiting high transfection activity *in vivo* confers advantages for establishing pharmaceutically acceptable preparations, and therefore facilitates liposome - based gene therapy.

transfected and incubated with 12 μg plasmid DNA complexed with lipid as described above (PCMV/IVS-luc+, luciferase reporter gene described above) for 4 hours at 37°C. The supernatant was then aspirated, fresh medium was added and the cells were incubated for 24 hours at 37°C. Cells were then harvested by washing with PBS (Ca/Mg free) and then suspended in lysis buffer for the luciferase assay, as described above.

Figure 5A shows that transfection of non-target cells, not over-expressing the HER-2 receptor, was inhibited by the addition of PEG-PE, even in the presence of the targeting ligand conjugated at the tip of PEG via the terminal maleimide residue. Figure 5B shows that transfection of target cells overexpressing the HER-2 receptor was also inhibited by the addition of PEG-PE, but the transfection activity was restored and augmented when the PEG-PE was conjugated to a targeting ligand, which recognizes the HER-2 receptor.

Comparison of Figures 5A and 5B indicates that the targeted immuno-CLDC were active in transfecting target cells much more efficiently than non-target cells. This result occurs because the addition of the ligand-carrying stabilizing agent (PEG-PE) conjugated to anti-HER-2-Fab'), which inhibits the transfection of non-target cells (Figure 5A) but augments transfection of the target cells (Figure 5B).

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

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8. The method of claim 1, wherein said step of contacting said nucleic acid with said organic polycation comprises contacting said organic polycation and said nucleic acid in a ratio ranging from about 0.05 to about 5.0 nmole organic polycation:μg nucleic acid.

9. The method of claim 1, wherein said method comprises the steps of: contacting an expression cassette with a polyamine selected from the group consisting of spermidine and spermine to produce a condensed expression cassette; and

combining said condensed expression cassette with a lipid comprising DDAB and cholesterol from about 2:1 to about 1:2 molar ratio to produce a lipid:expression cassette complex, wherein said lipid:expression cassette complex comprising said condensed expression cassette has an increased shelf life at about 4°C as compared to an identical lipid:expression cassette complex lacking said polyamine.

10. The method of claim 9, wherein said lipid complex is mixed with an amphipathic lipid attached to polyethylene glycol conjugated to a Fab' fragment of an antibody.

11. The method of claim 1, wherein said lipid:nucleic acid complex is lyophilized.

12. A method of increasing the shelf life of a lipid:nucleic acid complex, said method comprising the steps of:

combining a nucleic acid with a lipid comprising an amphiphilic cationic lipid to produce said lipid:nucleic acid complex; and

mixing said lipid:nucleic acid complex with a hydrophilic polymer, wherein said lipid:nucleic acid complex has increased shelf life as compared to an identical lipid:nucleic acid complex lacking said hydrophilic polymer.

13. The method of claim 12, wherein said hydrophilic polymer is selected from the group consisting of polyethylene glycol (PEG), polyethylene glycol derivatized with phosphatidyl ethanolamine (PEG-PE),

20. The method of claim 19, wherein said lipid comprises polyethylene glycol attached to a Fab' fragment of an antibody.

21. The method of claim 12, wherein said lipid:nucleic acid complex is lyophilized.

22. A method of transfecting a nucleic acid into a cell, said method comprising the step of contacting said cell with a lipid:nucleic acid complex, said lipid:nucleic acid complex comprising:

said nucleic acid contacted with an organic polycation to produce a condensed nucleic acid; and

a lipid comprising an amphiphilic cationic lipid, wherein contacting said cell with said lipid:nucleic acid complex transfects said nucleic acid into said cell.

23. The method of claim 22, wherein said lipid:nucleic acid complex is stored at a temperature of about 22°C or below.

24. The method of claim 22, wherein said organic polycation is selected from the group consisting of polyamine, polyammonium, and basic polyamino acid.

25. The method of claim 24, wherein said polyamine is selected from the group consisting of spermine and spermidine.

26. The method of claim 22, wherein said nucleic acid is DNA.

27. The method of claim 22, wherein said step of contacting said cell with said lipid:nucleic acid complex comprises systemic administration of said lipid:nucleic acid complex into a mammal.

28. The method of claim 22, wherein said step of contacting said cell with said lipid:nucleic acid complex comprises intravenous administration of said lipid:nucleic acid complex into a mammal.

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administration of said lipid:nucleic acid complex into a mammal.

36. The method of claim 31, wherein said step of contacting said cell with said lipid:nucleic acid complex comprises intravenous administration of said lipid:nucleic acid complex into a mammal.

37. The method of claim 31, wherein said method comprises the step of contacting said cell with a lipid:expression cassette complex, said lipid:expression cassette complex comprising:

an expression cassette;

said lipid comprising said amphiphilic cationic lipid; and

polyethylene glycol derivatized with phosphatidyl ethanolamine (PEG-PE), wherein said lipid:expression cassette complex is stored at a temperature of about 22°C or below.

38. The method of claim 37, wherein said lipid comprises polyethylene glycol attached to a Fab' fragment of an antibody.

39. A lipid:nucleic acid complex comprising:

a nucleic acid contacted with an organic polycation to produce a condensed nucleic acid; and

a lipid comprising an amphiphilic cationic lipid, wherein said lipid:nucleic acid complex has an increased shelf life as compared to an identical lipid:nucleic acid complex lacking said organic polycation.

40. The complex of claim 39, wherein said organic polycation is selected from the group consisting of polyamine, polyammonium, and basic polyamino acid.

41. The complex of claim 40, wherein said polyamine is selected from the group consisting of spermine and spermidine.

42. The complex of claim 39, wherein said nucleic acid is selected from the group consisting of DNA and RNA.

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50. The composition of claim 49, wherein said polyamine is selected from the group consisting of spermine and spermidine.

51. The composition of claim 48, wherein said nucleic acid is DNA.

52. The composition of claim 48, wherein the amount of said lipid and said nucleic acid is in a ratio ranging from about 1 to about 20 nmole lipid:μg nucleic acid.

53. The composition of claim 48, wherein said composition comprises:

an expression cassette contacted with a polyamine selected from the group consisting of spermidine and spermine to produce a condensed expression cassette; and

said lipid comprising said amphiphilic cationic lipid, wherein said composition has an increased shelf life at about 4°C as compared to an identical composition lacking said polyamine.

54. The composition of claim 53, wherein said lipid comprises polyethylene glycol attached to a Fab' fragment of an antibody.

55. A kit for preparing a lipid:nucleic acid complex, said kit comprising:

(i) a container containing a liposome;

(ii) a container containing a nucleic acid; and

(iii) a container containing a hydrophilic polymer, wherein the liposome and the nucleic acid are mixed to form the lipid:nucleic acid complex and wherein the lipid:nucleic acid complex is contacted with the hydrophilic polymer.

56. A kit of claim 55, wherein said hydrophilic polymer is derivatized with a targeting moiety.

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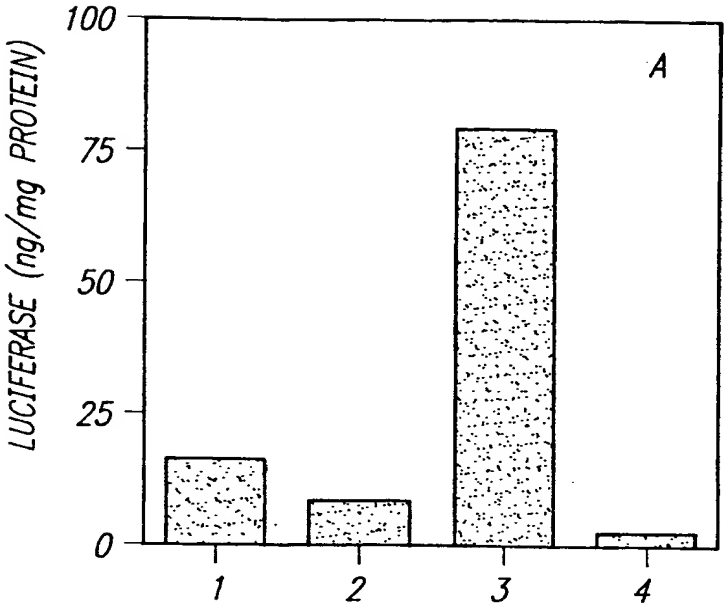

FIG. 1A

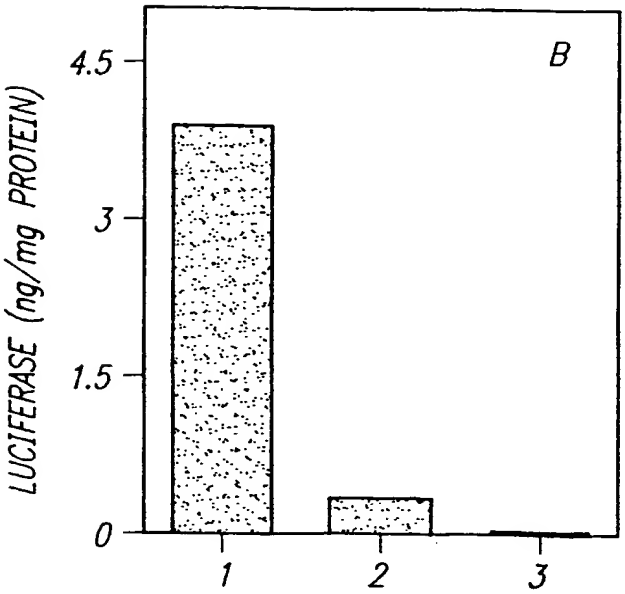

FIG. 1B

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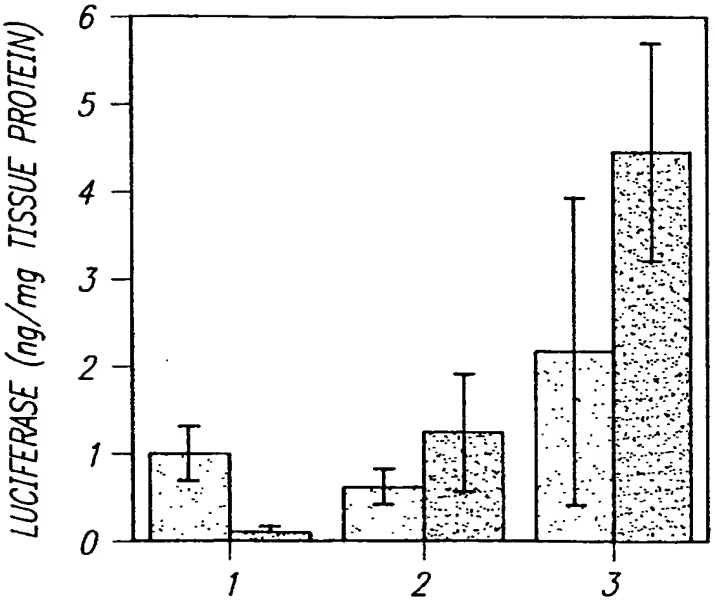

FIG. 4

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/20690

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 9/127, 31/30; C07H 21/00, 21/02, 21/04
US CL : 424/450; 514/44; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450; 435/172.3, 325, 375; 514/2, 44; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,459,127 A (FELGNER et al.) 17 October 1995, see entire document.	1-58

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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04 FEBRUARY 1998

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